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(57) Abstract

An apparatus for screening for translocation of a first protein of interest *in vivo* in a cell comprises (a) a total internal reflection member having a surface portion. If desired, the surface portion can be divided into separate and discrete segments. (b) A cell contacted to the surface portion by the plasma membrane of the cell, the protein having a fluorescent group conjugated thereto. If desired, different cells can be contacted to different ones of the separate and discrete segments. (c) A light source operatively associated with the total internal reflection member and positioned for directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, with the evanescent field being weaker in a second portion of the cell, the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell (i.e., less light as compared to the amount emitted when the same fluorescent group is in the first portion of the cell). (d) A light detector operatively associated with the total internal reflection member and configured to detect emitted light from the cell. The emission of more or less light from the cell indicates the translocation of the first protein between the first and second portions of the cell. Methods of using the apparatus, particularly for screening for binding or the disruption of binding between first and second proteins of interest, are also disclosed.

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METHOD AND APPARATUS FOR DETECTING BINDING INTERACTIONS *IN VIVO*

This invention was made with Government support under National Institutes of Health grants GM-48113 and GM-51457. The Government has certain rights to this invention.

Field of the Invention

The present invention concerns methods and apparatus for detecting binding interactions in cells. The methods and apparatus are particularly suitable for the high throughput screening of combinatorial libraries.

Background of the Invention

Biomolecular or combinatorial arrays have provided a means for the high throughput screening of chemical libraries. *See, e.g.,* U.S. Patent No. 5,143,854. A variety of specific techniques for carrying out the automated screening of such arrays have been developed, including the evanescent scanning of a pixel array. *See* U.S. Patent No. 5,633,724.

A disadvantage of combinatorial arrays is that they provide an *in vitro* rather than an *in vivo* assay. *In vitro* binding assays can seldom provide an accurate measure of how binding will actually occur *in vivo,* particularly for intracellular binding events, because the complexity of the intracellular environment is difficult to replicate outside of the cell. Of course, the ultimate application of many screening assays is to develop *in vivo* applications for the compounds being screened. Accordingly, there is a continued need for new *in vivo* screening techniques that can be readily adapted to automated or high throughput screening.

Summary of the Invention

A first aspect of the present invention is an apparatus for screening for translocation of a first protein of interest *in vivo* in a cell. The apparatus comprises:

(a) a total internal reflection member having a surface portion. If desired, the surface portion can be divided into separate and discrete segments.

(b) A cell contacted to the surface portion by the plasma membrane of the cell, the protein having a fluorescent group conjugated thereto. If desired, different cells can be contacted to different ones of the separate and discrete segments.

(c) A light source operatively associated with the total internal reflection member and positioned for directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, with the evanescent field being weaker in a second portion of the cell, the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell (*i.e.*, less light as compared to the amount emitted when the same fluorescent group is in the first portion of the cell).

(d) A light detector operatively associated with the total internal reflection member and configured to detect emitted light from the cell

The emission of more or less light from the cell indicates the translocation of the first protein between the first and second portions of the cell.

The cell or cells may further contain a second protein of interest located in either the first portion of the cell or the second portion of the cell, whereby the emission of more or less light from the cell indicates the presence or absence of specific binding between the first and second proteins of interest. When the second protein is located in the first portion of the cell, the emission of more light indicates the specific binding of the proteins of interest, and the emission of less light indicates the lack of such binding. When the second protein is located in the second portion of the cell, the emission of less light indicates the specific binding of the proteins of interest, and the emission of more light indicates the lack of such binding. First and second proteins of interest may be members of a specific binding pair. Either or both of the first and second proteins of interest may be expressed by a nucleic acid carried by the cell; either of the first and second proteins of interest may be a member of a library of compounds, with a different member of said library being expressed in cells

of different segments, while the other protein of interest is the same in the cells of different segments, to provide a way to rapidly screen the library of compounds.

A second aspect of the present invention is a method of detecting translocation of a first protein of interest within a cell. The method comprises:

(a) providing a total internal reflection member having a surface portion, with a cell contacted to the surface portion by the plasma membrane of the cell;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell; wherein the protein of interest has a fluorescent group conjugated thereto; the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell; and then

(c) detecting emitted light from the fluorescent group, with the emission of more or less light from the fluorescent group indicating the translocation of the first protein of interest between the first and second portions of the cell.

The method may be used with a second protein of interest as described in connection with the apparatus above. An analysis of a test compound (e.g., a member of a library of compounds as described below) may be carried out by administering a test compound to the cell to determine whether or not said test compound disrupts the binding of said first and second proteins of interest. The analysis may be made a quantitative analysis by repeating steps (a) through (c) with different cells at different concentrations of said test compound. The degree of binding or disruption of binding may then be determined at different concentrations of the test compound.

The methods and apparatus of the invention can be used on individual cells or for screening multiple cell populations, or libraries of cells or libraries of compounds, as described in greater detail below.

A further aspect of the present invention is a method of screening binding between a first protein of interest and a library of second proteins of interest within a plurality of cells. The method comprises:

(a) providing a total internal reflection member having a surface portion, the surface portion having a plurality of separate and discrete segments, with a cell contacted to each of the surface portion segments by the plasma membrane of the cells;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell;wherein one of the proteins of interest has a fluorescent group conjugated thereto, and the other of the proteins of interest is located in either the first portion of the cell or the second portion of the cell; and wherein one of the proteins of interest is the same in each of the cells; and the other of the proteins of interest is a different member of the library in cells contacted to different segments; with the fluorescent group emitting light when in the first portion of each of the cells and emitting less light when in the second portion of each of the cells;

and then

(c) detecting emitted light from each of the segments, with the presence or absence of emitted light indicating the presence or absence of specific binding between the proteins of interest in the cell in each of the segments.

A further aspect of the present invention is a method of screening a library of compounds for the ability to disrupt binding between first and second proteins of interest. The method comprises:

(a) providing a total internal reflection member having a surface portion, the surface portion having a plurality of separate and discrete segments, with a cell contacted to each of the surface portion segments by the plasma membrane thereof;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell;

wherein one of the proteins of interest has a fluorescent group conjugated thereto, and the other of the proteins of interest is located in either the first portion of the cell or the second portion of the cell;

the fluorescent group emitting light when in the first portion of each of the cells and emitting less light when in the second portion of each of the cells;

then

(c) administering a different member of the library of compounds to each of the separate and discrete segments (*e.g.*, by contacting a different compound to the cells, or by expressing a different compound from a different nucleic acid in each of said cells); and then

(d) detecting emitted light from the fluorescent group in the cells from each of the separate and discrete segment.

The presence or absence of emitted light from the fluorescent group indicates the disruption or lack of disruption of specific binding between the proteins of interest by the member of the library administered to the segment.

When screening libraries, the screening steps may be repeated with different members of the library until sufficient members of the library have been screened. It will also be appreciated that each cell may contain or be administered a subpopulation or subpool of the library, and that where a population or subpopulation is found to contain a compound having desired properties, the screening step may be repeated with additional subpopulations containing the desired compound until the population has been reduced to one or a sufficiently small number to permit identification of the compound desired.

The present invention is explained in greater detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 is a schematic illustration of an evanescent microscope of the invention, useful for carrying out a quantitative translocation analysis (QTA).

Figure 2 demonstrates a quantitative translocation analysis carried out with an apparatus of Figure 1.

Figure 3 demonstrates a photomultiplier simulation of a quantitative translocation analysis signal.

Figure 4 shows PAF induced plasma membrane translocation of C1-GFP measured by evanescent microscopy with an apparatus of Figure 1.

Figure 5 illustrates an apparatus of the present invention for screening a plurality of cells.

Detailed Description of the Preferred Embodiments

The term "contact" or contacted to", when used herein with respect to a cell or cells contacted to a total internal reflection member, means sufficiently close to be excitable by the evanescent wave. This means that, in a typical configuration, the the total internal relection member is less than 5 micrometer away from the cell membrane. Such contact may be direct or indirect through intervening materials (e.g, cell adhesion proteins or extracellular matrix).

Total internal reflection (TIR) members useful for carrying out the present invention include prisms, waveguides, fibers, and specialized microscope objectives. The member may be a single unitary element or a combination of elements (for example, a glass slide contacted to a prism by an intervening oil). In the case of waveguides and fibers, there may be several TIR surfaces. Additionally, the TIR surface can be on an optically transparent substrate surface such as a glass slide, optical film, or the like, that is optically coupled with the TIR element in a conventional manner, for example, using a refractive index matching oil or a compressible optical polymer such as those disclosed by Sjodin, "Optical interface means," PCT publication WO 90/05317, 1990. The substrate surface is preferably removable from the TIR element, and may even be disposable. If the test cell or cells are contacted directly to the TIR element, such as the prism, it may be necessary to clean or replace the prism before testing other cells , and the alignment of the prism will then have to be checked and possibly readjusted. Providing a removable substrate (e.g. by means of refractive index matching oil on, for example, a prism) eliminates or at least greatly reduces the cost and effort involved in ensuring that the prism is clean and aligned.

Light sources suitable for carrying out the present invention include, but are not limited to, lasers, LEDs, coherent frequency-converting devices (an example of

which is disclosed by Kozlovsky et at., "Resonator-enhanced frequency doubling in an extended-cavity diode laser," presented at Blue/Green Compact Lasers, New Orleans Feb. 1-5, 1993 and references therein), an array of surface emitting LEDs (Bare et al., "A simple surface-emitting LED array useful for developing free-space optical interconnects," I.E.E.E., Photon. Tech. Lett., Vol. 5, 172-175, 1993), and a suitable array of vertical-cavity surface-emitting lasers (VCSEL) where each polymer array pixel could have its own corresponding laser on the VCSEL array (Salah and Teich, Fundamentals of Photonics, Wiley-Interscience, New York, 1991, p. 638). Examples of molecular tag/light source pairs include CY5/HeNe laser, CY5/laser diode (e.g. Toshiba TOLD9410(s)), CY5/LED (e.g. Hewlett-Packard HMP8150), fluorescein/argon ion laser, and rhodamine/argon ion laser.

Any suitable light detector may be used to carry out the present invention. An example of a suitable detection system is shown in U.S. Patent No. 5,633,724 to King et al. As illustrated therein, an imaging system collects and images the optical signal through an optical filter and onto a two-dimensional array detector. Imaging systems can contain lenses or a coherent fiber bundle. The filter is chosen to transmit the optical signal and reject radiation at other frequencies. The detector is preferably a two dimensional detector such as CCD array, image intensified CCD, vidicon or video camera. An optional image intensifier, such as a Hamamatsu V4170U image intensifer, can be used in addition to detector if the optical signal is weak.

Cells used to carry out the present invention are typically eukaryotic cells, which may be yeast, plant, or animal cells. Yeast and animal cells, particularly mammalian cells, are currently preferred. Example plant cells include, but are not limited to, arabidopsis, tobacco, tomato and potato plant cells. Example animal cells include, but are not limited to, human, monkey, chimpanzee, rat, cat, dog, and mouse cells.

"Detectable groups" or "detectable proteins" used to carry out the present invention include fluorescent proteins, such as green fluorescent protein (GFP) and apoaequorin, including analogs and derivatives thereof. Green fluorescent protein is obtained from the jellyfish *Aequorea victoria* and has been expressed in a wide variety of microbial, plant, insect and mammalian cells. A. Crameri et al., *Nature Biotech.* 14, 315-319 (1996). Any detectable group may be employed, and other suitable detectable groups include other fluorophores or fluorescent indicators, such

as a fusion tag with any binding domain such as avidin, streptavidin and ligand binding domains of receptors. Coupling of biotin or other ligands to the fluorophore or indicator of interest may be achieved using a dextran matrix or other linker system. The detectable protein may be one which specifically binds a fluorophore, as in FLASH technology. Fluorescent detectable groups (including both fluorescent proteins and proteins that bind a separate fluorophore molecule thereto) are currently preferred.

"Internal structure" as used herein refers to a separate, discreet, identifiable component contained within a cell. The term "structure" as applied to the constituent parts of a cell is known (*see, e.g.,* R. Dyson, Cell Biology: A Molecular Approach, pg, 10 (2d ed. 1978)), and the term "internal structure" is intended to exclude external structures such as flagella and pili. Such internal structures are, in general, anatomical structures of the cell in which they are contained. Examples of internal structures include both structure located in the cytosol or cytoplasm outside of the nucleus (also called "cytoplasmic structures"), and structures located within the nucleus (also called "nuclear structures"). The nucleus itself including the nuclear membrane are internal structures. Structures located within the cytoplasm outside of the nucleus are currently preferred. Thus the term "internal structure" is specifically intended to include any non-uniformly distributed cellular component, including proteins, lipids, carbohydrates, nucleic acids, etc., and derivatives thereof.

"Library" as used herein refers to a collection of different compounds, typically organic compounds, assembled or gathered together in a form that they can be used together, either simultaneously or serially. The compounds may be small organic compounds or biopolymers, including proteins and peptides. The compounds may be encoded and produced by nucleic acids as intermediates, with the collection of nucleic acids also being referred to as a library. Where a nucleic acid library is used, it may be a random or partially random library, commonly known as a "combinatorial library" or "combinatorial chemistry library", or it may be a library obtained from a particular cell or organism, such as a genomic library or a cDNA library. Small organic molecules can be produced by combinatorial chemistry techniques as well. Thus in general, such libraries comprise are organic compounds, including but not limited oligomers, non-oligomers, or combinations thereof. Non-oligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics,

aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, prophyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins, oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide, herein) such as DNA and RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, sulfites, sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. *See, e.g.,* U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).

"Nucleic acid" as used herein refers to both DNA and RNA.

"Protein" as used herein is intended to include protein fragments, or peptides. Thus the term "protein" is used synonymously with the phrase "protein or fragment thereof" (for the purpose of brevity), particularly with reference to proteins that are "proteins of interest" or members of a specific binding pair. Protein fragments may or may not assume a secondary or tertiary structure. Protein fragments may be of any length, from 2, 3, 5 or 10 peptides in length up to 50, 100, or 200 peptides in length or more, up to the full length of the corresponding protein.

"Specifically binds" and "specific binding" as used herein includes but is not limited to stereospecific binding, electrostatic binding, or hydrophlic binding interactions. Thus, specifically binds and specific binding are exhibited by at least a two or three fold (or two or three times), greater apparent binding affinity between the binding partners as compared to other proteins or binding partners within the cell in which binding is being detected.

"Specific binding pair" refers to a pair of molecules (e.g., a pair of proteins) that specifically bind to one another. A pair of molecules that specifically bind to one another, which may be the same or different, are referred to as members of a specific binding pair. A protein that is a member of a specific binding pair may be a protein that has been previously determined to be a member of a specific binding pair or a

protein that is a putative member of a specific binding pair. Examples of the latter include members of a library, such as the products of a cDNA or combinatorial library, or a protein for which a binding partner has not yet been identified, where it is desired to identify a naturally occuring (*e.g.*, a product of a cDNA or genomic DNA library) or non-naturally occuring (*e.g.*, combinatorial) binding partner therefore.

"Translocation" as used herein refers to a change in distribution of a protein or conjugate (including a fusion protein) from one physical distribution within a cell to another, different, physical distribution within a cell. Preferably, translocation is from either a uniform or non-uniform distribution to a non-uniform distribution. Translocation could also be from a non-uniform to a uniform distribution. Translocation may be induced by any suitable means, such as by administration of a physical or chemical signal (*e.g.*, administration of a compound such as a phorbol ester or platelet activating factor (PAF)). Many signal transduction proteins are known to change their distribution after stimulation of the corresponding receptor (or other appropriate stimulus), and can be used to carry out the present invention. Often these translocation events are mediated by subdomains of such signalling proteins (*e.g.*, the C1 or C2 subdomains), and such subdomains can be used to carry out the present invention.

As noted above, the present invention provides a method of detecting a protein-protein interaction. The method comprises first providing a cell that contains a first heterologous conjugate and a second heterologous conjugate. The first heterologous conjugate comprises a first protein of interest conjugated to a detectable group. The second heterologous conjugate comprises a second protein of interest (which may be the same as or different from the first protein of interest) conjugated to a protein that specifically binds to an internal structure within the cell. The binding of the protein that specifically binds to an internal structure may be immediate, may be induced (as discussed below), or may be a prior binding in the case of a protein that is previously localized to or permanently located at the internal structure of interest. The two conjugates are preferably each present in the cell at a total concentration between about 1 or 10 nM to about 1 or 10 mM.

The presence or absence of binding of the detectable group to the internal structure is then detected, the presence of the binding indicating that the first and second proteins of interest specifically bind to one another. Detection may be by any

suitable means depending upon the detectable group employed, but preferably the detectable group is a fluorescent group and detection is carried out by optical or visual reading, which may be done manually, by an automated apparatus, or by combinations thereof.

If desired, the second heterologous conjugate can further comprise a detectable group, which detectable group is preferably different from the detectable group located on the first heterologous conjugate and fluoresces at a different wavelength therefrom. For example, both detectable groups could be a green fluorescent proteins, yet simply different mutants of green fluorescent protein that fluoresce at different wavelengths.

Either or both of the heterologous conjugates may be introduced directly in the cell by any suitable means, such as by electroporation or lipofection. In the alternative, when the heterologous conjugates are fusion proteins, a nucleic acid (typically a DNA) may be stable introduced into the cell (for example, as a plasmid), which nucleic acid includes a suitable promoter segment that controls and causes the expression of a nucleic acid encoding the fusion protein. Again, either or both of the fusion proteins may be produced in the cell in this matter.

Binding events in the instant invention may be direct or indirect binding events. Indirect binding events are those mediated through an intermediate, or bridging, molecule or conjugate. Administration of such a bridge molecule can be a signal to induce translocation (discussed below). For example, the bridging molecule may be a covalent conjugate of FK506 and cyclosporin, to cause the indirect binding of FKBP12 and cyclophilin (both conventionally cytosolic proteins) to one another. Either of the FKBP12 or the cyclophilin can be modified so that it binds to the plasma membreane, such as by lipidating the protein or forming a fusion protein with the transmembrane domain of a transmembrane protein.

Any internal structure as defined above can be used to carry out the present invention, as long as the binding of the detectable group to the internal structure provides a different detectable signal from the cell than when the detectable group is not bound to the internal structure. In one preferred embodiment the internal structure is contained in the cell cytoplasm. Examples of internal structures include, but are not limited to, plasma membrane, cytoskeleton (including but not limited to actin cytoskeleton, tubulin cytoskeleton, intermediate filaments, focal adhesions, etc.),

centromere, nucleus, mitochondria, endoplasmic reticulum, vacuoles, golgi apparatus, and chloroplasts. Preferably, the internal structure is either the plasma membrane or cortical cytoskeleton.

In a preferred embodiment of the invention, the protein that specifically binds to an internal structure is a translocatable protein. In this embodiment, the method further comprises the step of inducing translocation of the second heterologous conjugate prior to the detecting step. Induction of translocation may be carried out by any suitable means, such as by administration of a physical or chemical signal (e.g., administration of a compound such as a phorbol ester). Such a protein may be selected from the group consisting of cytosolic protein kinases, protein phosphatases, adapter proteins, cytoskeletal proteins, cytoskeleton associated proteins, GTP-binding proteins, plasma transmembrane proteins, plasma membrane associated proteins, β-arrestin, and visual arrestin (including fragments thereof that specifically bind to an internal structure). Preferably, the protein is a protein kinase C isoform or a fragment thereof that specifically binds to an internal structure, such as a C1 domain fragment or a C2 domain fragment of protein kinase C gamma (or other suitable protein kinase C), where the induction signal is administration of a phorbol ester. In addition, induction of translocation may be induced by stimulation of a receptor, such as a glutamate receptor, beta-adrenergic receptor, or PAF receptor, with a receptor agonist to induce a signaling step which in turn induces translocation. Finally, numerous proteins may be modified to make them translocatable by employing bridging molecules, as discussed above.

As noted above, in one embodiment of the invention the first and second proteins of interest may together comprise members of a specific binding pair. In this embodiment, the invention may further include the step of administering a test compound to the cell prior to the detecting step, wherein the absence of binding of the detectable group to the internal structure indicates that the test compound inhibits the binding of the members of the specific binding pair. Any test compound can be used, including peptides, oligonucleotides, expressed proteins, small organic molecules, known drugs and derivatives thereof, natural or non-natural compounds, etc. Administration of the test compound may be by any suitable means, including direct administration such as by electroporation or lipofection if the compound is not otherwise membrane permeable, or (where the test compound is a protein), by

introducing a heterologous nucleic acid that encodes and expresses the test compound into the cell. Such methods are useful for screening libraries of compounds for new compounds which disrupt the binding of a known binding pair.

Figure 1 is a schematic illustration of an evanescent microscope **10** of the invention, useful for carrying out a quantitative translocation analysis (QTA). A prism **11** is used as the total internal reflection (TIR) member. The prism is a dove prism made of crown glass (part number 01PDE005, Melles Griot, Irvine, CA). Excitation light **12** (argon ion laser at 488 nm) for generating the evanescent field is provided by an Enterprise argon-ion laser **13** obtained from Coherent Inc., Moutainview, CA. A glass cover slip **14** made of crown glass has cells adhered thereto, and is contacted to the dove prism with an intervening coating of oil (Immersionsol N518 from Zeiss, Inc.). The angle of the laser light into the TIR member is adjusted to provide an evanescent field in first and (weakly) second portions of the cells, as explained above. A Zeiss Axioskop microscope stand is used as a stand for a Princeton Instrument CCD 1300-Y **15** (Trenton, NJ) photon detection system, which is in turn connected to a personal computer programmed with appropriate data collection and analysis software.

Figure 2 demonstrates a quantitative translocation analysis carried out with an apparatus of Figure 1. RBL cells expressing C2-GFP were stimulated with PAF. The fluorescence signal average **20** was measured from 6 cells. Each cell had relative signal increases that ranged from 3 to 6 fold.

Figure 3 demonstrates a photomultiplier simulation of a quantitative translocation analysis signal, with the same data alayzed as for the single cells in Figure 2 above. The average relative fluorescence increase **21** for the entire field of view (simulating what a photomultiplyer would encounter) is illustrated. The field of view was approximately 0.15 mm in diameter.

Figure 4 illustrates PAF induced plasma membrane translocation of C1-GFP measured by evanescent microscopy with an apparatus of Figure 1. The relative increase in fluorescence intensity **22** for an average of six cells is shown at 3 second time-points.

Figure 5 illustrates an apparatus of the present invention for simultaneously screening a plurality of cells. The apparatus comprises a light source **31**, a prism **32**, a substrate **33** positioned on top of the prism and optically contacted to the prism by

means of an oil 44 with a similar index of refraction to the substrate and the prism, an optical detection system 36 and a computer 37. The light source, prism, and substrate may be the same as the light source, prism and substrate described in Example 1 above. The substrate is divided into segments by a rigid polymer grid 40 that is secured to the substrate by means of an adhesive. Cells 45 are adhered to the substrate in each of the discrete regions, as described above. Excitation light 46 produces an evanescent field in first regions of the cells which in turn generates a light signal 47 from the cells that is detectable or distinguishable by detection system 36 when the fluorescent group is in the first region of the cells. The various components of the system can be used in like manner to that described in U.S. Patent No. 5,633,724 (the disclosure of which is incorporated by reference herein in its entirety), except that cells are employed in contrast to compounds. The discrete regions on the substrate can be formed by any suitable means, such as by adhering a physical barrier such as a grid to the substrate, simply adhering cells in different regions, forming wells, channels, grooves or other physical barriers in the substrate, etc. The apparatus of Figure 5 can be used for the simultaneous screening of a plurality of cells, which may be the same or different. Specific screening techniques that may be employed are as follows:

Screen 1: Identification of chemical libraries, drug collections, peptides, antisense and other compounds that interfere with particular signaling events. Many signal transduction events result in the translocation of proteins between two cell portions of which one portion is near the plasma membrane. Any such signaling process that leads to such a translocation event can be screened using the evanescent wave method (i.e. calcium (C2-domain), diacylglycerol signals (C1-domains), tyrosine phosphorylation (SH2-domains), phosphatidylinositol polyphosphate signals (different PH-domains), phosphorylation of seven-transmembrane receptors (?-arrestin). Establishment of cell lines that express the respective translocatable fluorescently conjugated signaling protein or protein domain is recommended.

Useful also as secondary screen of compounds that were identified by in vitro binding assays and which may or may not function as inhibitors in the cellular environment.

Screen 2: Screen for supressors or enhancers of known signaling pathways. Screening cDNA libraries for expressed proteins that either suppress or

enhance the signal transduction event monitored by the evanescent wave translocation assay.

Screen 3: Screen for mutant cells that show suppressed or enhanced signal transduction events. This application includes the screening of randomly mutated cells that show different signaling responses and the subsequent identification of the mutated gene.

Screen 4: Identification of ligands of orphan receptors. Many G-protein coupled, tyrosine kinase and other receptors have unknown ligands. A large set of such ligands or drugs that may bind to the orphan receptor can be identified with this method.

Using a translocatable fluorescent protein downstream of the receptor such as C1, C2, PH-domains or β-arrestin to monitor evanescent wave intensity changes.

Screen 5: Identification of novel binding partners of a known protein. Binding interaction between the known protein X and a large number of unknown proteins Y are screened. Protein X is made as a conjugate with a fluorescent group or fluorescent protein while a library of the proteins Y is made that are each coupled to a protein or protein domain that can translocate between the two cell portions in response to addition of a drug, receptor stimuli or other procedure. A cell line is then made using the fluorescent protein X. The library of proteins Y with conjugated translocatable groups is then transfected into these fluorescent cell lines using pooling of the library or random transfection. Binding partners are then identified that show a drug or stimulus induced change in fluorescence intensity. Fluorescene intensities from individual cells or from segments of the evanescent wave apparatus surface are recorded. Either single cells are selected for analysis or segments of cells when pool assays are used.

The inverse screen is also possible with a library of proteins that are each coupled to a fluorescently labeled protein and a single known protein that is conjugated to a protein or protein domain that can translocate between the two cell portions.

Screen 6: Identification of compounds that suppress or enhance known protein-protein binding interactions. This screen is carried out with libraries of compounds of various types, as described above.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

What is Claimed is:

1. An apparatus for screening for translocation of a first protein of interest *in vivo* in a cell, comprising:

(a) a total internal reflection member having a surface portion, with

(b) a cell contacted to said surface portion by the plasma membrane of said cell, said cell containing said first protein of interest, said protein of interest having a fluorescent group conjugated thereto;

(c) a light source operatively associated with said total internal reflection member and positioned for directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell, said fluorescent group emitting light when in said first portion of said cell and emitting less light when in said second portion of said cell; and

(d) a light detector operatively associated with said total internal reflection member and configured to detect emitted light from said cell, whereby the emission of more or less light from said cell indicates the translocation of said protein between said first and second portions of said cell.

2. An apparatus according to claim 1, wherein said light source comprises a coherent light source.

3. An apparatus according to claim 1, wherein said total internal reflection member comprises a prism.

4. An apparatus according to claim 1, wherein said light detector comprises a CCD camera.

5. An apparatus according to claim 1 wherein said protein having said fluorescent group conjugated thereto is a first protein of interest, said cell further containing a second protein of interest located in either said first portion of said cell or said second portion of said cell, whereby the emission of more or less light from said

cell indicates the presence or absence of specific binding between said first and second proteins of interest.

6. An apparatus according to claim 1, wherein said first and second proteins are members of a specific binding pair.

7. An apparatus for screening binding events between first and second proteins of interest in a plurality of cells, comprising:

(a) a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments formed thereon;

(b) cells contacted to each of said surface portion segments by the plasma membrane of said cells;

(c) a light source operatively associated with said total internal reflection member and positioned for directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of each of said cells adjacent said plasma membrane, said evanescent field being weaker in a second portion of each of said cells;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cells or said second portion of said cells;

said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells; and

(d) a light detector operatively associated with said total internal reflection member and configured to detect emitted light from each of said segments.

8. An apparatus according to claim 7, wherein said light source comprises a coherent light source.

9. An apparatus according to claim 7, wherein said total internal reflection member comprises a prism.

10. An apparatus according to claim 7, wherein said light detector comprises a CCD camera.

11. An apparatus according to claim 7, wherein said first and second proteins of interest are members of a specific binding pair.

12. A method of detecting translocation of a first protein of interest within a cell, comprising:

(a) providing a total internal reflection member having a surface portion, with a cell contacted to said surface portion by the plasma membrane of said cell;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell; wherein said protein of interest has a fluorescent group conjugated thereto; said fluorescent group emitting light when in said first portion of said cell and emitting less light when in said second portion of said cell; and then

(c) detecting emitted light from said fluorescent group, with the emission of more or less light from said fluorescent group indicating the translocation of said first protein of interest between said first and second portions of said cell.

13. A method according to claim 12, wherein said source light is coherent light.

14. A method according to claim 12, wherein said total internal reflection member comprises a prism.

15. A method according to claim 12, wherein said detecting step is carried out with a CCD camera.

16. A method according to claim 12, said cell further containing a second protein of interest, said second protein of interest located in either said first portion of said cell or said second portion of said cell, wherein the emission of more or less light

from said fluorescent group indicates the presence or absence of specific binding between said first and second proteins of interest.

17. A method according to claim 16, wherein said first and second proteins of interest are members of a specific binding pair.

18. A method according to claim 16, further comprising the step of administering a test compound to said cell to determine whether or not said test compound disrupts the binding of said first and second proteins of interest.

19. A method according to claim 18, further comprising the step of repeating steps (a) through (c) at different concentrations of said test compound.

20. A method of screening binding between a first protein of interest and a library of second proteins of interest within a plurality of cells, comprising:

(a) providing a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments, with a cell contacted to each of said surface portion segments by the plasma membrane of said cells;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cell or said second portion of said cell;

and wherein one of said proteins of interest is the same in each of said cells; and the other of said proteins of interest is a different member of said library in cells contacted to different segments;

with said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells;

and then

(c) detecting emitted light from each of said segments, with the presence or absence of emitted light indicating the presence or absence of specific binding between said proteins of interest in the cell in each of said segments.

21. A method according to claim 20, wherein said source light is coherent light.

22. A method according to claim 20, wherein said total internal reflection member comprises a prism.

23. A method according to claim 20, wherein said detecting step is carried out with a CCD camera.

24. A method according to claim 20, further comprising the step of repeating steps (a) to (c) with a subpopulation of said library.

25. A method of screening a library of compounds for the ability to disrupt binding between first and second proteins of interest, comprising:

(a) providing a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments, with a cell contacted to each of said surface portion segments by the plasma membrane thereof;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cell or said second portion of said cell;

said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells;

then

(c) administering a different member of said library of compounds to each of said separate and discrete segments; and then

(d) detecting emitted light from said fluorescent group in said cells from each of said separate and discrete segments, with the presence or absence of emitted light from said fluorescent group indicating the disruption or lack of disruption of binding between said proteins of interest by the member of said library administered to said segment.

26. A method according to claim 25, wherein said source light is coherent light.

27. A method according to claim 25, wherein said total internal reflection member comprises a prism.

28. A method according to claim 25, wherein said detecting step is carried out with a CCD camera.

29. A method according to claim 25, further comprising the step of repeating steps (a) through (d) with a subpopulation of said library.

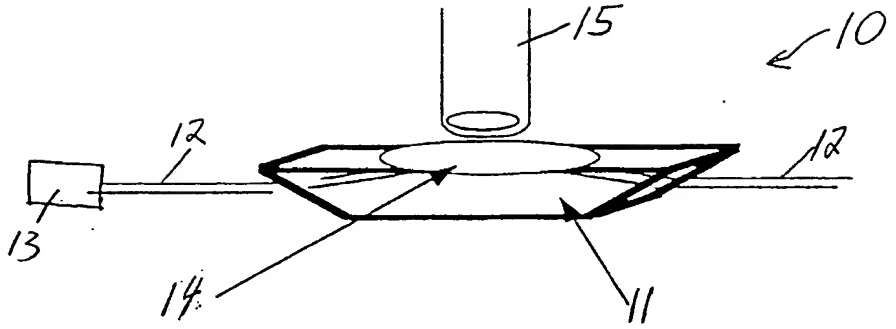
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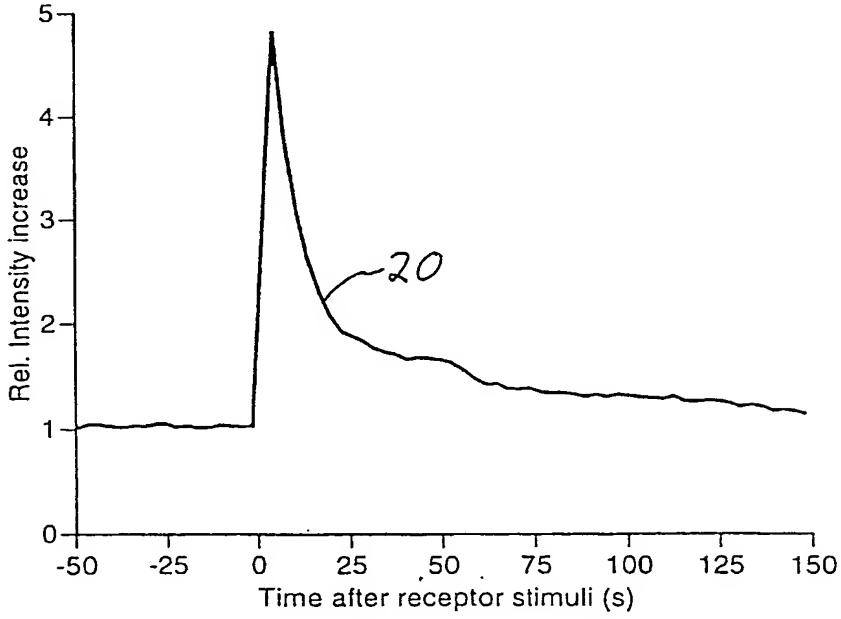
FIG 1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Time after receptor stimuli (s)

FIG. 2

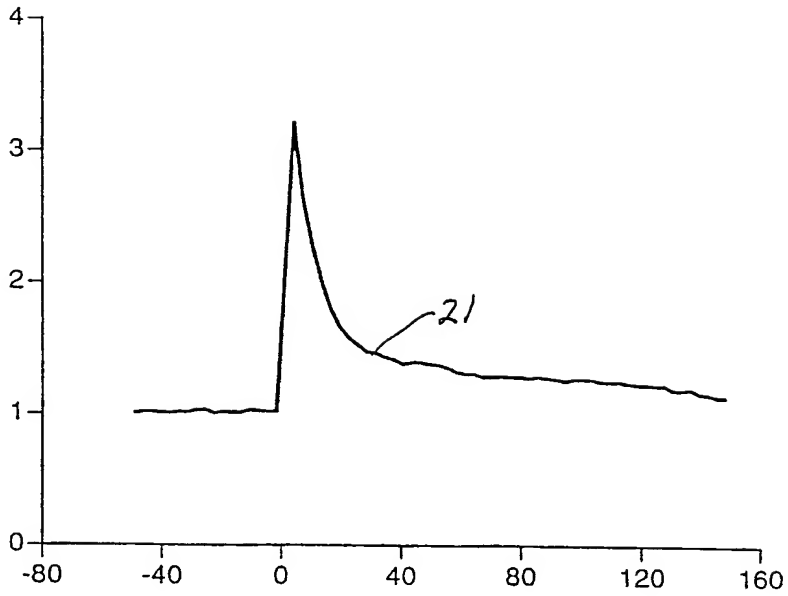
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FIG 3

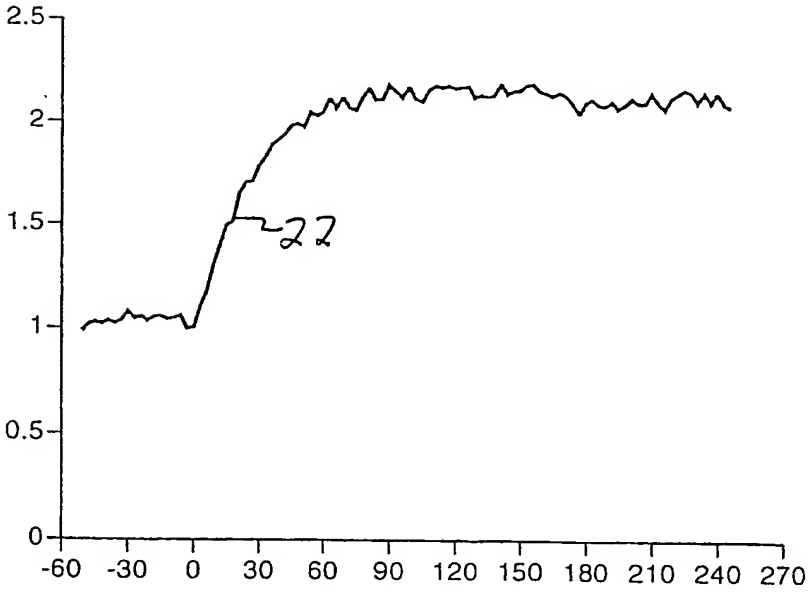
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FIG 4

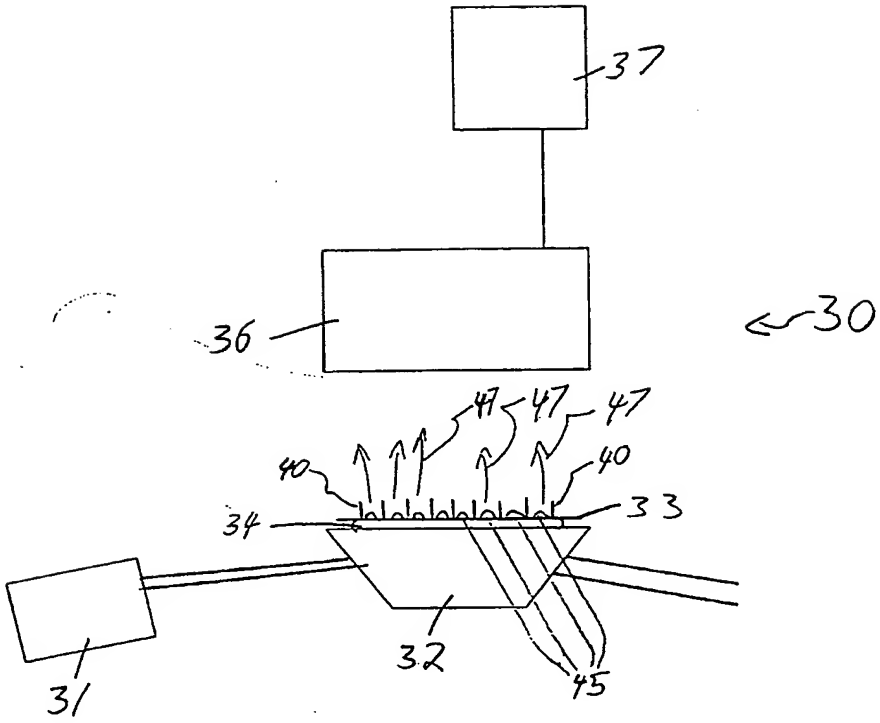

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19696

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :G01N 33/53, 21/55, 21/41; G01B 9/02
US CL :435/7.2, 7.1, 4; 356/445, 136, 352
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.2, 7.1, 4; 356/445, 136, 352

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EAST and CASONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,633,724 A (KING et al) 27 May 1997, col. 3, line 62 up to col. 7, line 10.	1-29
Y	US 5,143,854 A (PIRRUNG et al) 01 September 1992, col.8, line 17 up to col. 15, line 35.	1-29
Y	W0 97/27212 A1 (RIGEL PHARMACEUTICALS, INC.) 31 July 1997, entire document.	1-29
Y	PERRETTI, M. et al. Investigation of rat mast cell degranulation using flow cytometry. Journal of Pharmacological Methods. 1990, Vol. 23, pages 187-194, entire document.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search 11 DECEMBER 1999	Date of mailing of the international search report 04 FEB 2000
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International application No.
PCT/US99/19696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIYAWAKI, A. et al. Fluroescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature. 28 August 1997, Vol. 388, pages 882-887, entire document.	1-29
A	XU, X. et al. Detection of programmed cell death using fluorescence energy transfer. Nucleic Acids Research. 1988, Vol. 26, No. 8, pages 2034-2035, entire document.	1-29
A	Wendland, B. et al. A novel flourescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. The Journal of Cell Biology. December 1996, Vol. 135, No. 6, Part 1, pages 1485-1500, entire document.	1-29

CORRECTED VERSION*

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(54) Title: METHOD AND APPARATUS FOR DETECTING BINDING INTERACTIONS *IN VIVO*

(57) Abstract

An apparatus for screening for translocation of a first protein of interest *in vivo* in a cell comprises (a) a total internal reflection member having a surface portion. If desired, the surface portion can be divided into separate and discrete segments. (b) A cell contacted to the surface portion by the plasma membrane of the cell, the protein having a fluorescent group conjugated thereto. If desired, different cells can be contacted to different ones of the separate and discrete segments. (c) A light source operatively associated with the total internal reflection member and positioned for directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, with the evanescent field being weaker in a second portion of the cell, the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell (i.e., less light as compared to the amount emitted when the same fluorescent group is in the first portion of the cell). (d) A light detector operatively associated with the total internal reflection member and configured to detect emitted light from the cell. The emission of more or less light from the cell indicates the translocation of the first protein between the first and second portions of the cell. Methods of using the apparatus, particularly for screening for binding or the disruption of binding between first and second proteins of interest, are also disclosed.

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METHOD AND APPARATUS FOR DETECTING BINDING INTERACTIONS *IN VIVO*

This invention was made with Government support under National Institutes of Health grants GM-48113 and GM-51457. The Government has certain rights to this invention.

Field of the Invention

The present invention concerns methods and apparatus for detecting binding interactions in cells. The methods and apparatus are particularly suitable for the high throughput screening of combinatorial libraries.

Background of the Invention

Biomolecular or combinatorial arrays have provided a means for the high throughput screening of chemical libraries. *See, e.g.*, U.S. Patent No. 5,143,854. A variety of specific techniques for carrying out the automated screening of such arrays have been developed, including the evanescent scanning of a pixel array. *See* U.S. Patent No. 5,633,724.

A disadvantage of combinatorial arrays is that they provide an *in vitro* rather than an *in vivo* assay. *In vitro* binding assays can seldom provide an accurate measure of how binding will actually occur *in vivo,* particularly for intracellular binding events, because the complexity of the intracellular environment is difficult to replicate outside of the cell. Of course, the ultimate application of many screening assays is to develop *in vivo* applications for the compounds being screened. Accordingly, there is a continued need for new *in vivo* screening techniques that can be readily adapted to automated or high throughput screening.

Summary of the Invention

A first aspect of the present invention is an apparatus for screening for translocation of a first protein of interest *in vivo* in a cell. The apparatus comprises:

(a) a total internal reflection member having a surface portion. If desired, the surface portion can be divided into separate and discrete segments.

(b) A cell contacted to the surface portion by the plasma membrane of the cell, the protein having a fluorescent group conjugated thereto. If desired, different cells can be contacted to different ones of the separate and discrete segments.

(c) A light source operatively associated with the total internal reflection member and positioned for directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, with the evanescent field being weaker in a second portion of the cell, the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell (*i.e.*, less light as compared to the amount emitted when the same fluorescent group is in the first portion of the cell).

(d) A light detector operatively associated with the total internal reflection member and configured to detect emitted light from the cell

The emission of more or less light from the cell indicates the translocation of the first protein between the first and second portions of the cell.

The cell or cells may further contain a second protein of interest located in either the first portion of the cell or the second portion of the cell, whereby the emission of more or less light from the cell indicates the presence or absence of specific binding between the first and second proteins of interest. When the second protein is located in the first portion of the cell, the emission of more light indicates the specific binding of the proteins of interest, and the emission of less light indicates the lack of such binding. When the second protein is located in the second portion of the cell, the emission of less light indicates the specific binding of the proteins of interest, and the emission of more light indicates the lack of such binding. First and second proteins of interest may be members of a specific binding pair. Either or both of the first and second proteins of interest may be expressed by a nucleic acid carried by the cell; either of the first and second proteins of interest may be a member of a library of compounds, with a different member of said library being expressed in cells

of different segments, while the other protein of interest is the same in the cells of different segments, to provide a way to rapidly screen the library of compounds.

A second aspect of the present invention is a method of detecting translocation of a first protein of interest within a cell. The method comprises:

(a) providing a total internal reflection member having a surface portion, with a cell contacted to the surface portion by the plasma membrane of the cell;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell; wherein the protein of interest has a fluorescent group conjugated thereto; the fluorescent group emitting light when in the first portion of the cell and emitting less light when in the second portion of the cell; and then

(c) detecting emitted light from the fluorescent group, with the emission of more or less light from the fluorescent group indicating the translocation of the first protein of interest between the first and second portions of the cell.

The method may be used with a second protein of interest as described in connection with the apparatus above. An analysis of a test compound (e.g., a member of a library of compounds as described below) may be carried out by administering a test compound to the cell to determine whether or not said test compound disrupts the binding of said first and second proteins of interest. The analysis may be made a quantitative analysis by repeating steps (a) through (c) with different cells at different concentrations of said test compound. The degree of binding or disruption of binding may then be determined at different concentrations of the test compound.

The methods and apparatus of the invention can be used on individual cells or for screening multiple cell populations, or libraries of cells or libraries of compounds, as described in greater detail below.

A further aspect of the present invention is a method of screening binding between a first protein of interest and a library of second proteins of interest within a plurality of cells. The method comprises:

(a) providing a total internal reflection member having a surface portion, the surface portion having a plurality of separate and discrete segments, with a cell contacted to each of the surface portion segments by the plasma membrane of the cells;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell;wherein one of the proteins of interest has a fluorescent group conjugated thereto, and the other of the proteins of interest is located in either the first portion of the cell or the second portion of the cell; and wherein one of the proteins of interest is the same in each of the cells; and the other of the proteins of interest is a different member of the library in cells contacted to different segments; with the fluorescent group emitting light when in the first portion of each of the cells and emitting less light when in the second portion of each of the cells;

and then

(c) detecting emitted light from each of the segments, with the presence or absence of emitted light indicating the presence or absence of specific binding between the proteins of interest in the cell in each of the segments.

A further aspect of the present invention is a method of screening a library of compounds for the ability to disrupt binding between first and second proteins of interest. The method comprises:

(a) providing a total internal reflection member having a surface portion, the surface portion having a plurality of separate and discrete segments, with a cell contacted to each of the surface portion segments by the plasma membrane thereof;

(b) directing a source light into the member to produce an evanescent field adjacent the surface portion, with the evanescent field extending into a first portion of the cell adjacent the plasma membrane, the evanescent field being weaker in a second portion of the cell;

wherein one of the proteins of interest has a fluorescent group conjugated thereto, and the other of the proteins of interest is located in either the first portion of the cell or the second portion of the cell;

the fluorescent group emitting light when in the first portion of each of the cells and emitting less light when in the second portion of each of the cells;

then

(c) administering a different member of the library of compounds to each of the separate and discrete segments (*e.g.*, by contacting a different compound to the cells, or by expressing a different compound from a different nucleic acid in each of said cells); and then

(d) detecting emitted light from the fluorescent group in the cells from each of the separate and discrete segment.

The presence or absence of emitted light from the fluorescent group indicates the disruption or lack of disruption of specific binding between the proteins of interest by the member of the library administered to the segment.

When screening libraries, the screening steps may be repeated with different members of the library until sufficient members of the library have been screened. It will also be appreciated that each cell may contain or be administered a subpopulation or subpool of the library, and that where a population or subpopulation is found to contain a compound having desired properties, the screening step may be repeated with additional subpopulations containing the desired compound until the population has been reduced to one or a sufficiently small number to permit identification of the compound desired.

The present invention is explained in greater detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 is a schematic illustration of an evanescent microscope of the invention, useful for carrying out a quantitative translocation analysis (QTA).

Figure 2 demonstrates a quantitative translocation analysis carried out with an apparatus of Figure 1.

Figure 3 demonstrates a photomultiplier simulation of a quantitative translocation analysis signal.

Figure 4 shows PAF induced plasma membrane translocation of C1-GFP measured by evanescent microscopy with an apparatus of Figure 1.

Figure 5 illustrates an apparatus of the present invention for screening a plurality of cells.

Detailed Description of the Preferred Embodiments

The term "contact" or contacted to", when used herein with respect to a cell or cells contacted to a total internal reflection member, means sufficiently close to be excitable by the evanescent wave. This means that, in a typical configuration, the the total internal relection member is less than 5 micrometer away from the cell membrane. Such contact may be direct or indirect through intervening materials (e.g, cell adhesion proteins or extracellular matrix).

Total internal reflection (TIR) members useful for carrying out the present invention include prisms, waveguides, fibers, and specialized microscope objectives. The member may be a single unitary element or a combination of elements (for example, a glass slide contacted to a prism by an intervening oil). In the case of waveguides and fibers, there may be several TIR surfaces. Additionally, the TIR surface can be on an optically transparent substrate surface such as a glass slide, optical film, or the like, that is optically coupled with the TIR element in a conventional manner, for example, using a refractive index matching oil or a compressible optical polymer such as those disclosed by Sjodin, "Optical interface means," PCT publication WO 90/05317, 1990. The substrate surface is preferably removable from the TIR element, and may even be disposable. If the test cell or cells are contacted directly to the TIR element, such as the prism, it may be necessary to clean or replace the prism before testing other cells , and the alignment of the prism will then have to be checked and possibly readjusted. Providing a removable substrate (e.g. by means of refractive index matching oil on, for example, a prism) eliminates or at least greatly reduces the cost and effort involved in ensuring that the prism is clean and aligned.

Light sources suitable for carrying out the present invention include, but are not limited to, lasers, LEDs, coherent frequency-converting devices (an example of

which is disclosed by Kozlovsky et at., "Resonator-enhanced frequency doubling in an extended-cavity diode laser," presented at Blue/Green Compact Lasers, New Orleans Feb. 1-5, 1993 and references therein), an array of surface emitting LEDs (Bare et al., "A simple surface-emitting LED array useful for developing free-space optical interconnects," I.E.E.E., Photon. Tech. Lett., Vol. 5, 172-175, 1993), and a suitable array of vertical-cavity surface-emitting lasers (VCSEL) where each polymer array pixel could have its own corresponding laser on the VCSEL array (Salah and Teich, Fundamentals of Photonics, Wiley-Interscience, New York, 1991, p. 638). Examples of molecular tag/light source pairs include CY5/HeNe laser, CY5/laser diode (e.g. Toshiba TOLD9410(s)), CY5/LED (e.g. Hewlett-Packard HMP8150), fluorescein/argon ion laser, and rhodamine/argon ion laser.

Any suitable light detector may be used to carry out the present invention. An example of a suitable detection system is shown in U.S. Patent No. 5,633,724 to King et al. As illustrated therein, an imaging system collects and images the optical signal through an optical filter and onto a two-dimensional array detector. Imaging systems can contain lenses or a coherent fiber bundle. The filter is chosen to transmit the optical signal and reject radiation at other frequencies. The detector is preferably a two dimensional detector such as CCD array, image intensified CCD, vidicon or video camera. An optional image intensifier, such as a Hamamatsu V4170U image intensifer, can be used in addition to detector if the optical signal is weak.

Cells used to carry out the present invention are typically eukaryotic cells, which may be yeast, plant, or animal cells. Yeast and animal cells, particularly mammalian cells, are currently preferred. Example plant cells include, but are not limited to, arabidopsis, tobacco, tomato and potato plant cells. Example animal cells include, but are not limited to, human, monkey, chimpanzee, rat, cat, dog, and mouse cells.

"Detectable groups" or "detectable proteins" used to carry out the present invention include fluorescent proteins, such as green fluorescent protein (GFP) and apoaequorin, including analogs and derivatives thereof. Green fluorescent protein is obtained from the jellyfish *Aequorea victoria* and has been expressed in a wide variety of microbial, plant, insect and mammalian cells. A. Crameri et al., *Nature Biotech.* **14**, 315-319 (1996). Any detectable group may be employed, and other suitable detectable groups include other fluorophores or fluorescent indicators, such

as a fusion tag with any binding domain such as avidin, streptavidin and ligand binding domains of receptors. Coupling of biotin or other ligands to the fluorophore or indicator of interest may be achieved using a dextran matrix or other linker system. The detectable protein may be one which specifically binds a fluorophore, as in FLASH technology. Fluorescent detectable groups (including both fluorescent proteins and proteins that bind a separate fluorophore molecule thereto) are currently preferred.

"Internal structure" as used herein refers to a separate, discreet, identifiable component contained within a cell. The term "structure" as applied to the constituent parts of a cell is known (*see, e.g.*, R. Dyson, Cell Biology: A Molecular Approach, pg, 10 (2d ed. 1978)), and the term "internal structure" is intended to exclude external structures such as flagella and pili. Such internal structures are, in general, anatomical structures of the cell in which they are contained. Examples of internal structures include both structure located in the cytosol or cytoplasm outside of the nucleus (also called "cytoplasmic structures"), and structures located within the nucleus (also called "nuclear structures"). The nucleus itself including the nuclear membrane are internal structures. Structures located within the cytoplasm outside of the nucleus are currently preferred. Thus the term "internal structure" is specifically intended to include any non-uniformly distributed cellular component, including proteins, lipids, carbohydrates, nucleic acids, etc., and derivatives thereof.

"Library" as used herein refers to a collection of different compounds, typically organic compounds, assembled or gathered together in a form that they can be used together, either simultaneously or serially. The compounds may be small organic compounds or biopolymers, including proteins and peptides. The compounds may be encoded and produced by nucleic acids as intermediates, with the collection of nucleic acids also being referred to as a library. Where a nucleic acid library is used, it may be a random or partially random library, commonly known as a "combinatorial library" or "combinatorial chemistry library", or it may be a library obtained from a particular cell or organism, such as a genomic library or a cDNA library. Small organic molecules can be produced by combinatorial chemistry techniques as well. Thus in general, such libraries comprise are organic compounds, including but not limited oligomers, non-oligomers, or combinations thereof. Non-oligomers include a wide variety of organic molecules, such as heterocyclics, aromatics, alicyclics,

aliphatics and combinations thereof, comprising steroids, antibiotics, enzyme inhibitors, ligands, hormones, drugs, alkaloids, opioids, benzodiazepenes, terpenes, prophyrins, toxins, catalysts, as well as combinations thereof. Oligomers include peptides (that is, oligopeptides) and proteins, oligonucleotides (the term oligonucleotide also referred to simply as "nucleotide, herein) such as DNA and RNA, oligosaccharides, polylipids, polyesters, polyamides, polyurethanes, polyureas, polyethers, poly (phosphorus derivatives) such as phosphates, phosphonates, phosphoramides, phosphonamides, phosphites, phosphinamides, etc., poly (sulfur derivatives) such as sulfones, sulfonates, sulfites, sulfonamides, sulfenamides, etc., where for the phosphorous and sulfur derivatives the indicated heteroatom for the most part will be bonded to C, H, N, O or S, and combinations thereof. *See, e.g.,* U.S. Patent No. 5,565,324 to Still et al., U.S. Patent No. 5,284,514 to Ellman et al., U.S. Patent No. 5,445,934 to Fodor et al. (the disclosures of all United States patents cited herein are to be incorporated herein by reference in their entirety).

"Nucleic acid" as used herein refers to both DNA and RNA.

"Protein" as used herein is intended to include protein fragments, or peptides. Thus the term "protein" is used synonymously with the phrase "protein or fragment thereof" (for the purpose of brevity), particularly with reference to proteins that are "proteins of interest" or members of a specific binding pair. Protein fragments may or may not assume a secondary or tertiary structure. Protein fragments may be of any length, from 2, 3, 5 or 10 peptides in length up to 50, 100, or 200 peptides in length or more, up to the full length of the corresponding protein.

"Specifically binds" and "specific binding" as used herein includes but is not limited to stereospecific binding, electrostatic binding, or hydrophlic binding interactions. Thus, specifically binds and specific binding are exhibited by at least a two or three fold (or two or three times), greater apparent binding affinity between the binding partners as compared to other proteins or binding partners within the cell in which binding is being detected.

"Specific binding pair" refers to a pair of molecules (e.g., a pair of proteins) that specifically bind to one another. A pair of molecules that specifically bind to one another, which may be the same or different, are referred to as members of a specific binding pair. A protein that is a member of a specific binding pair may be a protein that has been previously determined to be a member of a specific binding pair or a

protein that is a putative member of a specific binding pair. Examples of the latter include members of a library, such as the products of a cDNA or combinatorial library, or a protein for which a binding partner has not yet been identified, where it is desired to identify a naturally occuring (*e.g.*, a product of a cDNA or genomic DNA library) or non-naturally occuring (*e.g.*, combinatorial) binding partner therefore.

"Translocation" as used herein refers to a change in distribution of a protein or conjugate (including a fusion protein) from one physical distribution within a cell to another, different, physical distribution within a cell. Preferably, translocation is from either a uniform or non-uniform distribution to a non-uniform distribution. Translocation could also be from a non-uniform to a uniform distribution. Translocation may be induced by any suitable means, such as by administration of a physical or chemical signal (*e.g.*, administration of a compound such as a phorbol ester or platelet activating factor (PAF)). Many signal transduction proteins are known to change their distribution after stimulation of the corresponding receptor (or other appropriate stimulus), and can be used to carry out the present invention. Often these translocation events are mediated by subdomains of such signalling proteins (*e.g.*, the C1 or C2 subdomains), and such subdomains can be used to carry out the present invention.

As noted above, the present invention provides a method of detecting a protein-protein interaction. The method comprises first providing a cell that contains a first heterologous conjugate and a second heterologous conjugate. The first heterologous conjugate comprises a first protein of interest conjugated to a detectable group. The second heterologous conjugate comprises a second protein of interest (which may be the same as or different from the first protein of interest) conjugated to a protein that specifically binds to an internal structure within the cell. The binding of the protein that specifically binds to an internal structure may be immediate, may be induced (as discussed below), or may be a prior binding in the case of a protein that is previously localized to or permanently located at the internal structure of interest. The two conjugates are preferably each present in the cell at a total concentration between about 1 or 10 nM to about 1 or 10 mM.

The presence or absence of binding of the detectable group to the internal structure is then detected, the presence of the binding indicating that the first and second proteins of interest specifically bind to one another. Detection may be by any

suitable means depending upon the detectable group employed, but preferably the detectable group is a fluorescent group and detection is carried out by optical or visual reading, which may be done manually, by an automated apparatus, or by combinations thereof.

If desired, the second heterologous conjugate can further comprise a detectable group, which detectable group is preferably different from the detectable group located on the first heterologous conjugate and fluoresces at a different wavelength therefrom. For example, both detectable groups could be a green fluorescent proteins, yet simply different mutants of green fluorescent protein that fluoresce at different wavelengths.

Either or both of the heterologous conjugates may be introduced directly in the cell by any suitable means, such as by electroporation or lipofection. In the alternative, when the heterologous conjugates are fusion proteins, a nucleic acid (typically a DNA) may be stable introduced into the cell (for example, as a plasmid), which nucleic acid includes a suitable promoter segment that controls and causes the expression of a nucleic acid encoding the fusion protein. Again, either or both of the fusion proteins may be produced in the cell in this matter.

Binding events in the instant invention may be direct or indirect binding events. Indirect binding events are those mediated through an intermediate, or bridging, molecule or conjugate. Administration of such a bridge molecule can be a signal to induce translocation (discussed below). For example, the bridging molecule may be a covalent conjugate of FK506 and cyclosporin, to cause the indirect binding of FKBP12 and cyclophilin (both conventionally cytosolic proteins) to one another. Either of the FKBP12 or the cyclophilin can be modified so that it binds to the plasma membreane, such as by lipidating the protein or forming a fusion protein with the transmembrane domain of a transmembrane protein.

Any internal structure as defined above can be used to carry out the present invention, as long as the binding of the detectable group to the internal structure provides a different detectable signal from the cell than when the detectable group is not bound to the internal structure. In one preferred embodiment the internal structure is contained in the cell cytoplasm. Examples of internal structures include, but are not limited to, plasma membrane, cytoskeleton (including but not limited to actin cytoskeleton, tubulin cytoskeleton, intermediate filaments, focal adhesions, etc.),

centromere, nucleus, mitochondria, endoplasmic reticulum, vacuoles, golgi apparatus, and chloroplasts. Preferably, the internal structure is either the plasma membrane or cortical cytoskeleton.

In a preferred embodiment of the invention, the protein that specifically binds to an internal structure is a translocatable protein. In this embodiment, the method further comprises the step of inducing translocation of the second heterologous conjugate prior to the detecting step. Induction of translocation may be carried out by any suitable means, such as by administration of a physical or chemical signal (e.g., administration of a compound such as a phorbol ester). Such a protein may be selected from the group consisting of cytosolic protein kinases, protein phosphatases, adapter proteins, cytoskeletal proteins, cytoskeleton associated proteins, GTP-binding proteins, plasma transmembrane proteins, plasma membrane associated proteins, β-arrestin, and visual arrestin (including fragments thereof that specifically bind to an internal structure). Preferably, the protein is a protein kinase C isoform or a fragment thereof that specifically binds to an internal structure, such as a C1 domain fragment or a C2 domain fragment of protein kinase C gamma (or other suitable protein kinase C), where the induction signal is administration of a phorbol ester. In addition, induction of translocation may be induced by stimulation of a receptor, such as a glutamate receptor, beta-adrenergic receptor, or PAF receptor, with a receptor agonist to induce a signaling step which in turn induces translocation. Finally, numerous proteins may be modified to make them translocatable by employing bridging molecules, as discussed above.

As noted above, in one embodiment of the invention the first and second proteins of interest may together comprise members of a specific binding pair. In this embodiment, the invention may further include the step of administering a test compound to the cell prior to the detecting step, wherein the absence of binding of the detectable group to the internal structure indicates that the test compound inhibits the binding of the members of the specific binding pair. Any test compound can be used, including peptides, oligonucleotides, expressed proteins, small organic molecules, known drugs and derivatives thereof, natural or non-natural compounds, etc. Administration of the test compound may be by any suitable means, including direct administration such as by electroporation or lipofection if the compound is not otherwise membrane permeable, or (where the test compound is a protein), by

introducing a heterologous nucleic acid that encodes and expresses the test compound into the cell. Such methods are useful for screening libraries of compounds for new compounds which disrupt the binding of a known binding pair.

Figure 1 is a schematic illustration of an evanescent microscope **10** of the invention, useful for carrying out a quantitative translocation analysis (QTA). A prism **11** is used as the total internal reflection (TIR) member. The prism is a dove prism made of crown glass (part number 01PDE005, Melles Griot, Irvine, CA). Excitation light **12** (argon ion laser at 488 nm) for generating the evanescent field is provided by an Enterprise argon-ion laser **13** obtained from Coherent Inc., Moutainview, CA. A glass cover slip **14** made of crown glass has cells adhered thereto, and is contacted to the dove prism with an intervening coating of oil (Immersionsol N518 from Zeiss, Inc.). The angle of the laser light into the TIR member is adjusted to provide an evanescent field in first and (weakly) second portions of the cells, as explained above. A Zeiss Axioskop microscope stand is used as a stand for a Princeton Instrument CCD 1300-Y **15** (Trenton, NJ) photon detection system, which is in turn connected to a personal computer programmed with appropriate data collection and analysis software.

Figure 2 demonstrates a quantitative translocation analysis carried out with an apparatus of Figure 1. RBL cells expressing C2-GFP were stimulated with PAF. The fluorescence signal average **20** was measured from 6 cells. Each cell had relative signal increases that ranged from 3 to 6 fold.

Figure 3 demonstrates a photomultiplier simulation of a quantitative translocation analysis signal, with the same data alayzed as for the single cells in Figure 2 above. The average relative fluorescence increase **21** for the entire field of view (simulating what a photomultiplyer would encounter) is illustrated. The field of view was approximately 0.15 mm in diameter.

Figure 4 illustrates PAF induced plasma membrane translocation of C1-GFP measured by evanescent microscopy with an apparatus of Figure 1. The relative increase in fluorescence intensity **22** for an average of six cells is shown at 3 second time-points.

Figure 5 illustrates an apparatus of the present invention for simultaneously screening a plurality of cells. The apparatus comprises a light source **31**, a prism **32**, a substrate **33** positioned on top of the prism and optically contacted to the prism by

means of an oil 44 with a similar index of refraction to the substrate and the prism, an optical detection system 36 and a computer 37. The light source, prism, and substrate may be the same as the light source, prism and substrate described in Example 1 above. The substrate is divided into segments by a rigid polymer grid 40 that is secured to the substrate by means of an adhesive. Cells 45 are adhered to the substrate in each of the discrete regions, as described above. Excitation light 46 produces an evanescent field in first regions of the cells which in turn generates a light signal 47 from the cells that is detectable or distinguishable by detection system 36 when the fluorescent group is in the first region of the cells. The various components of the system can be used in like manner to that described in U.S. Patent No. 5,633,724 (the disclosure of which is incorporated by reference herein in its entirety), except that cells are employed in contrast to compounds. The discrete regions on the substrate can be formed by any suitable means, such as by adhering a physical barrier such as a grid to the substrate, simply adhering cells in different regions, forming wells, channels, grooves or other physical barriers in the substrate, etc. The apparatus of Figure 5 can be used for the simultaneous screening of a plurality of cells, which may be the same or different. Specific screening techniques that may be employed are as follows:

Screen 1: Identification of chemical libraries, drug collections, peptides, antisense and other compounds that interfere with particular signaling events. Many signal transduction events result in the translocation of proteins between two cell portions of which one portion is near the plasma membrane. Any such signaling process that leads to such a translocation event can be screened using the evanescent wave method (i.e. calcium (C2-domain), diacylglycerol signals (C1-domains), tyrosine phosphorylation (SH2-domains), phosphatidylinositol polyphosphate signals (different PH-domains), phosphorylation of seven-transmembrane receptors (?-arrestin). Establishment of cell lines that express the respective translocatable fluorescently conjugated signaling protein or protein domain is recommended.

Useful also as secondary screen of compounds that were identified by in vitro binding assays and which may or may not function as inhibitors in the cellular environment.

Screen 2: Screen for supressors or enhancers of known signaling pathways. Screening cDNA libraries for expressed proteins that either suppress or

enhance the signal transduction event monitored by the evanescent wave translocation assay.

Screen 3: Screen for mutant cells that show suppressed or enhanced signal transduction events. This application includes the screening of randomly mutated cells that show different signaling responses and the subsequent identification of the mutated gene.

Screen 4: Identification of ligands of orphan receptors. Many G-protein coupled, tyrosine kinase and other receptors have unknown ligands. A large set of such ligands or drugs that may bind to the orphan receptor can be identified with this method.

Using a translocatable fluorescent protein downstream of the receptor such as C1, C2, PH-domains or β-arrestin to monitor evanescent wave intensity changes.

Screen 5: Identification of novel binding partners of a known protein. Binding interaction between the known protein X and a large number of unknown proteins Y are screened. Protein X is made as a conjugate with a fluorescent group or fluorescent protein while a library of the proteins Y is made that are each coupled to a protein or protein domain that can translocate between the two cell portions in response to addition of a drug, receptor stimuli or other procedure. A cell line is then made using the fluorescent protein X. The library of proteins Y with conjugated translocatable groups is then transfected into these fluorescent cell lines using pooling of the library or random transfection. Binding partners are then identified that show a drug or stimulus induced change in fluorescence intensity. Fluorescene intensities from individual cells or from segments of the evanescent wave apparatus surface are recorded. Either single cells are selected for analysis or segments of cells when pool assays are used.

The inverse screen is also possible with a library of proteins that are each coupled to a fluorescently labeled protein and a single known protein that is conjugated to a protein or protein domain that can translocate between the two cell portions.

Screen 6: Identification of compounds that suppress or enhance known protein-protein binding interactions. This screen is carried out with libraries of compounds of various types, as described above.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

What is Claimed is:

1. An apparatus for screening for translocation of a first protein of interest *in vivo* in a cell, comprising:

(a) a total internal reflection member having a surface portion, with

(b) a cell contacted to said surface portion by the plasma membrane of said cell, said cell containing said first protein of interest, said protein of interest having a fluorescent group conjugated thereto;

(c) a light source operatively associated with said total internal reflection member and positioned for directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell, said fluorescent group emitting light when in said first portion of said cell and emitting less light when in said second portion of said cell; and

(d) a light detector operatively associated with said total internal reflection member and configured to detect emitted light from said cell, whereby the emission of more or less light from said cell indicates the translocation of said protein between said first and second portions of said cell.

2. An apparatus according to claim 1, wherein said light source comprises a coherent light source.

3. An apparatus according to claim 1, wherein said total internal reflection member comprises a prism.

4. An apparatus according to claim 1, wherein said light detector comprises a CCD camera.

5. An apparatus according to claim 1 wherein said protein having said fluorescent group conjugated thereto is a first protein of interest, said cell further containing a second protein of interest located in either said first portion of said cell or said second portion of said cell, whereby the emission of more or less light from said

cell indicates the presence or absence of specific binding between said first and second proteins of interest.

6. An apparatus according to claim 1, wherein said first and second proteins are members of a specific binding pair.

7. An apparatus for screening binding events between first and second proteins of interest in a plurality of cells, comprising:

(a) a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments formed thereon;

(b) cells contacted to each of said surface portion segments by the plasma membrane of said cells;

(c) a light source operatively associated with said total internal reflection member and positioned for directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of each of said cells adjacent said plasma membrane, said evanescent field being weaker in a second portion of each of said cells;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cells or said second portion of said cells;

said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells; and

(d) a light detector operatively associated with said total internal reflection member and configured to detect emitted light from each of said segments.

8. An apparatus according to claim 7, wherein said light source comprises a coherent light source.

9. An apparatus according to claim 7, wherein said total internal reflection member comprises a prism.

10. An apparatus according to claim 7, wherein said light detector comprises a CCD camera.

11. An apparatus according to claim 7, wherein said first and second proteins of interest are members of a specific binding pair.

12. A method of detecting translocation of a first protein of interest within a cell, comprising:

(a) providing a total internal reflection member having a surface portion, with a cell contacted to said surface portion by the plasma membrane of said cell;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell; wherein said protein of interest has a fluorescent group conjugated thereto; said fluorescent group emitting light when in said first portion of said cell and emitting less light when in said second portion of said cell; and then

(c) detecting emitted light from said fluorescent group, with the emission of more or less light from said fluorescent group indicating the translocation of said first protein of interest between said first and second portions of said cell.

13. A method according to claim 12, wherein said source light is coherent light.

14. A method according to claim 12, wherein said total internal reflection member comprises a prism.

15. A method according to claim 12, wherein said detecting step is carried out with a CCD camera.

16. A method according to claim 12, said cell further containing a second protein of interest, said second protein of interest located in either said first portion of said cell or said second portion of said cell, wherein the emission of more or less light

from said fluorescent group indicates the presence or absence of specific binding between said first and second proteins of interest.

17. A method according to claim 16, wherein said first and second proteins of interest are members of a specific binding pair.

18. A method according to claim 16, further comprising the step of administering a test compound to said cell to determine whether or not said test compound disrupts the binding of said first and second proteins of interest.

19. A method according to claim 18, further comprising the step of repeating steps (a) through (c) at different concentrations of said test compound.

20. A method of screening binding between a first protein of interest and a library of second proteins of interest within a plurality of cells, comprising:

(a) providing a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments, with a cell contacted to each of said surface portion segments by the plasma membrane of said cells;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cell or said second portion of said cell;

and wherein one of said proteins of interest is the same in each of said cells; and the other of said proteins of interest is a different member of said library in cells contacted to different segments;

with said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells;

and then

(c) detecting emitted light from each of said segments, with the presence or absence of emitted light indicating the presence or absence of specific binding between said proteins of interest in the cell in each of said segments.

21. A method according to claim 20, wherein said source light is coherent light.

22. A method according to claim 20, wherein said total internal reflection member comprises a prism.

23. A method according to claim 20, wherein said detecting step is carried out with a CCD camera.

24. A method according to claim 20, further comprising the step of repeating steps (a) to (c) with a subpopulation of said library.

25. A method of screening a library of compounds for the ability to disrupt binding between first and second proteins of interest, comprising:

(a) providing a total internal reflection member having a surface portion, said surface portion having a plurality of separate and discrete segments, with a cell contacted to each of said surface portion segments by the plasma membrane thereof;

(b) directing a source light into said member to produce an evanescent field adjacent said surface portion, with said evanescent field extending into a first portion of said cell adjacent said plasma membrane, said evanescent field being weaker in a second portion of said cell;

wherein one of said proteins of interest has a fluorescent group conjugated thereto, and the other of said proteins of interest is located in either said first portion of said cell or said second portion of said cell;

said fluorescent group emitting light when in said first portion of each of said cells and emitting less light when in said second portion of each of said cells;

then

(c) administering a different member of said library of compounds to each of said separate and discrete segments; and then

(d) detecting emitted light from said fluorescent group in said cells from each of said separate and discrete segments, with the presence or absence of emitted light from said fluorescent group indicating the disruption or lack of disruption of binding between said proteins of interest by the member of said library administered to said segment.

26. A method according to claim 25, wherein said source light is coherent light.

27. A method according to claim 25, wherein said total internal reflection member comprises a prism.

28. A method according to claim 25, wherein said detecting step is carried out with a CCD camera.

29. A method according to claim 25, further comprising the step of repeating steps (a) through (d) with a subpopulation of said library.

FIG. 1

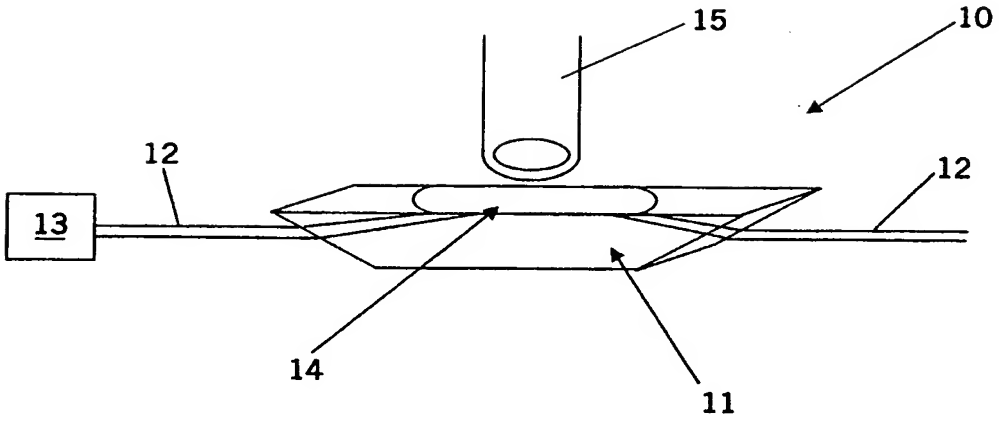
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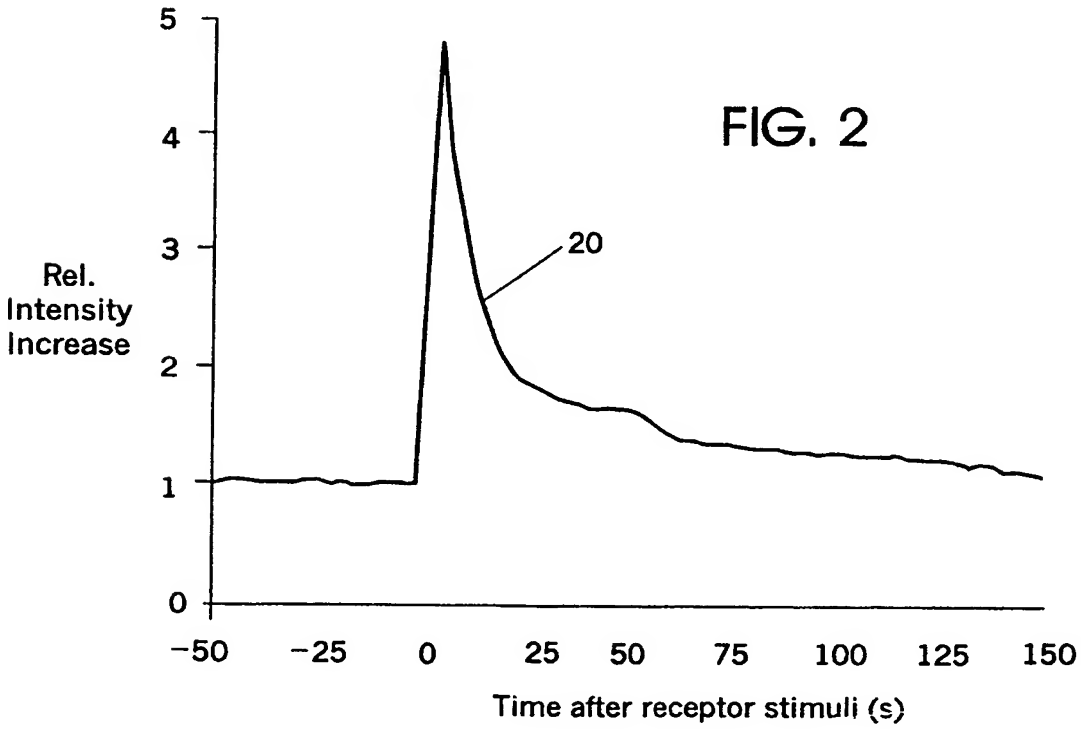
FIG. 2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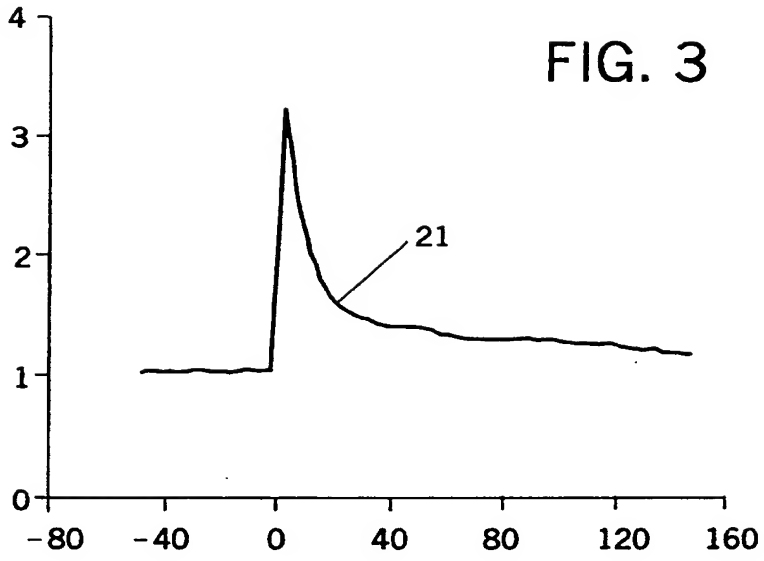
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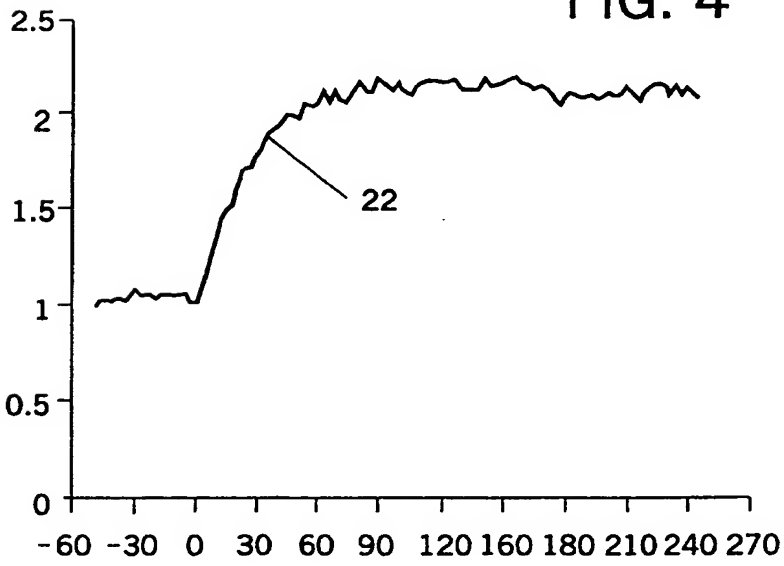
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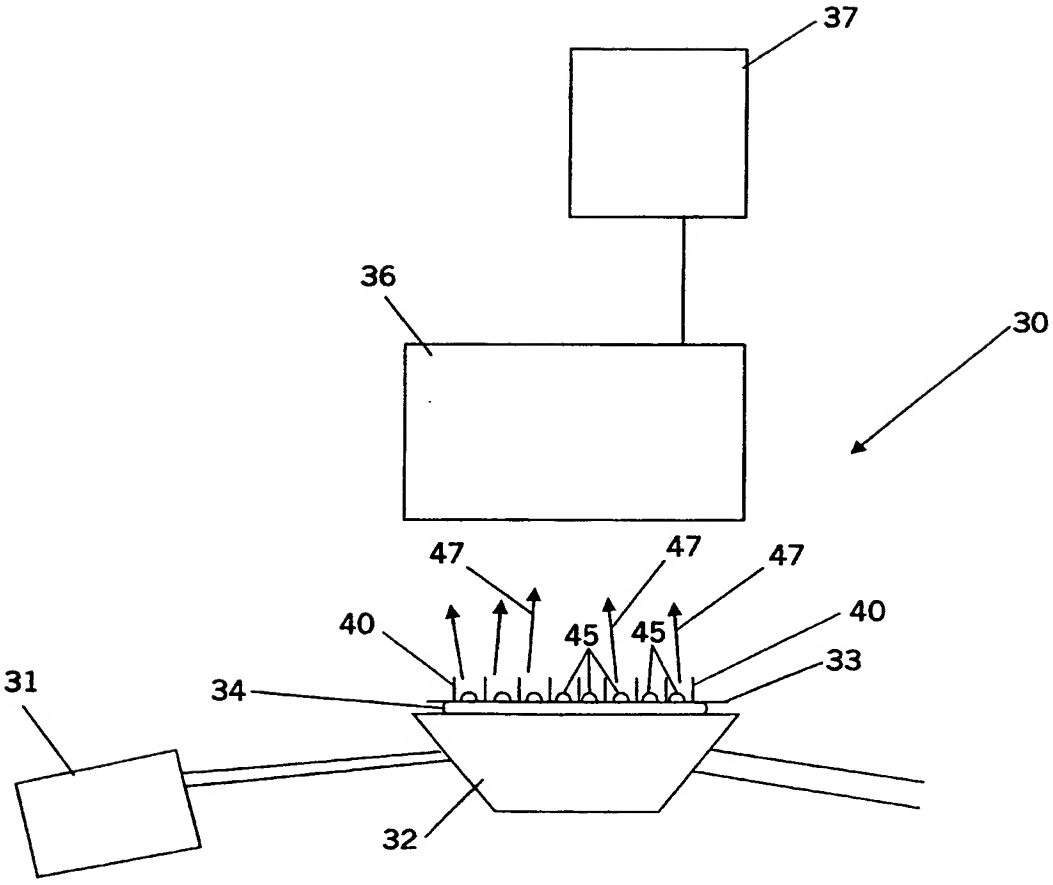
FIG. 4

FIG. 5



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19696

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53, 21/55, 21/41; G01B 9/02
US CL :435/7.2, 7.1, 4; 356/445, 136, 352
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 7.1, 4; 356/445, 136, 352

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST and CASONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,633,724 A (KING et al) 27 May 1997, col. 3, line 62 up to col. 7, line 10.	1-29
Y	US 5,143,854 A (PIRRUNG et al) 01 September 1992, col.8, line 17 up to col. 15, line 35.	1-29
Y	WO 97/27212 A1 (RIGEL PHARMACEUTICALS, INC.) 31 July 1997, entire document.	1-29
Y	PERRETTI, M. et al. Investigation of rat mast cell degranulation using flow cytometry. Journal of Pharmacological Methods. 1990, Vol. 23, pages 187-194, entire document.	1-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 DECEMBER 1999	Date of mailing of the international search report 04 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer T. WESSENDORF Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19696

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIYAWAKI, A. et al. Fluroescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature. 28 August 1997, Vol. 388, pages 882-887, entire document.	1-29
A	XU, X. et al. Detection of programmed cell death using fluorescence energy transfer. Nucleic Acids Research. 1988, Vol. 26, No. 8, pages 2034-2035, entire document.	1-29
A	Wendland, B. et al. A novel flourescence-activated cell sorter-based screen for yeast endocytosis mutants identifies a yeast homologue of mammalian eps15. The Journal of Cell Biology. December 1996, Vol. 135, No. 6, Part 1, pages 1485-1500, entire document.	1-29